

DESCRIPTION

PROCESS FOR PREPARING VARIANT OF ERYSIPELOTHRIX  
RHUSIOPATHIAE SURFACE PROTECTIVE ANTIGEN IN E. COLI

5 TECHNICAL FIELD

The present invention relates to a process for preparing a variant of Erysipelothrix rhusiopathiae surface protective antigen (hereinafter also referred to as "SpaA") with Escherichia coli as a host. More particularly, the present invention relates to a process for preparing a variant of SpaA or of a shortened form of SpaA (hereinafter also referred to as " $\Delta$ SpaA"), in which a portion of SpaA is deleted, with introduction of amino acid substitution wherein said variant may be expressed as insoluble inclusion bodies when expressed within the cells of E. coli, and to a variant of a recombinant SpaA or  $\Delta$ SpaA protein obtained by said process.

BACKGROUND ART

Porcine erysipelas is a swine disease caused by infection with Erysipelothrix rhusiopathiae wherein infected swine suffers from symptoms such as sepsis in acute cases, hives in subacute cases, or endocarditis and arthritis in chronic cases. Around 3,000 swine per year have been reported to have the disease which is a great deal of damage to a stockbreeder. Erysipelothrix

rhusiopathiae is pathogenic either to food animals such as wild boar, whales, chickens and turkeys in addition to swine and is specified as one of supervisory infectious diseases in the Protective Act of Livestock Diseases.

5 Porcine erysipelas is also zoonosis that causes erysipeloid in human and is of importance in view of meat hygiene. There are a number of serotypes in Erysipelothrix rhusiopathiae, among which serotypes 1 and 2 cause most of porcine erysipelas in swine.

10 For protection of porcine erysipelas infections, there have hitherto been used attenuated live vaccines, i.e. freeze-dried live vaccines prepared by using Koganei strain which is an attenuated strain of Erysipelothrix rhusiopathiae prepared by subculturing virulent  
15 Erysipelothrix rhusiopathiae in a medium supplemented with acriflavine for a long period; inactivated vaccines, i.e. bacteria vaccines prepared by treating a culture of virulent Erysipelothrix rhusiopathiae with formalin and rendering the whole cells and extracellular products be  
20 adsorbed to aluminum hydroxide gel; and component vaccines, i.e. ones comprising a fraction of non-purified surface proteins of the cells extracted from the whole cells with an aqueous alkali solution. Attenuated live vaccines are  
25 thought to be much less costly since they may be efficacious with only one administration in a small amount. However, it

is indicated they are also problematic in that they are pathogenic in mice to induce arthritis, that they exhibit severe side effects in swine with a low antibody level or SPF swine, and that the vaccine strain is isolated from the lesion of swine suffering from porcine erysipelas.

As a new type of vaccines, research and development is on-going for recombinant vaccines by the use of genetic recombination technique. Galan and Timony immunized mice with a lysate of E. coli transfected with a recombinant phage expressing genes from a part of Erysipelothrix rhusiopathiae genome and performed a challenge test with Erysipelothrix rhusiopathiae to observe that 14 to 17% of the immunized mice escaped from death after infection. Furthermore, they revealed that the proteins encoded by the genes are ones having molecular weight 66, 64, and 43 kDa from their reactivity with an immune serum against the lysate and demonstrated that these proteins could be protective antigens to Erysipelothrix rhusiopathiae infection (see e.g. Non-patent reference 1).

Makino et al. expressed a gene coding for a surface protein of a molecular weight 64 kDa (named "SpaA") from type 2 Erysipelothrix rhusiopathiae Tama 96 strain in E. coli, immunized mice with live cells of the resulting recombinant E. coli, and performed a challenge test with Erysipelothrix rhusiopathiae to demonstrate that SpaA

protein had protective activity to infection. They also revealed that SpaA protein had a sequence of 606 amino acid residues wherein a signal peptide consisting of 29 amino acids is at its N-terminal and eight homologous sequences of repeat, each repeat consisting of 20 amino acids excepting the 8th repeat which consists of 19 amino acids, are at its C-terminal (see e.g. Non-patent reference 2).

Imada et al. investigated SpaA protein from type 1 Fujisawa strain corresponding to the above SpaA protein and a gene encoding said protein to reveal that SpaA protein from type 1 Fujisawa strain is one with a molecular weight 69 kDa that has a sequence of 626 amino acid residues with one more, i.e. nine, homologous sequences of repeat at its C-terminal, as compared to the type 2 SpaA protein, with the 9th repeat consisting of 19 amino acids. They demonstrated that a fusion protein of a full-length SpaA, SpaA with deletion of the homologous sequences of repeat at the C-terminal, or SpaA with deletion of a portion of the N-terminal and the homologous sequences of repeat at the C-terminal, with a histidine hexamer, exhibited a protective effect to infection (see e.g. Non-patent references 3 and 4).

Watanabe et al. also reported that a polypeptide of 46.5 kDa prepared by deleting the homologous sequences of repeat at the C-terminal and a secretion signal sequence at the N-terminal from Erysipelothrix rhusiopathiae SpaA

protein could be a protective antigen to infection (46.5 kDa protective antigen; named "46.5 KPA") (see e.g. Patent reference 1).

On the other hand, promotion of productivity of a candidate protein for vaccine has been attempted. For instance, there is a report that 46.5 KPA could successfully be expressed for secretion out of the cells using Brevibacillus choshinensis as a host cell (see e.g. Patent reference 2). With this expression system, about 50% of an expressed protein becomes insoluble due to coagulation in culture. According to the report, purification of said insolubilized 46.5 KPA was performed by filtering a culture with ultrafiltration membrane, suspending the insoluble materials recovered on the membrane in an alkaline solution, and recovering the solubilized 46.5 KPA. Thus, this purification process requires at least three steps: (1) condensation through ultrafiltration under neutral to weak alkaline condition (pH 7 to 9.5); (2) recovery of a filtration fraction through ultrafiltration under strong alkaline condition (pH 10.0 to 12.0); and (3) purification of the ultrafiltration fraction by ion exchange chromatography.

When SpaA gene is expressed in E. coli, most of the protein may be expressed as a soluble protein and hence the purification process for insoluble materials as

described above may not be applied. A culture may contain, other than SpaA protein of interest, various contaminants such as cell debris of E. coli, components from a culture medium, metabolic products produced while culture, etc. It is not easy to efficiently recover and purify the soluble SpaA protein of interest from such admixtures of contaminants. In general, a vaccine for animals, unlike a vaccine for human, would not be accepted by a stockbreeder unless it is low priced as well as in high purity and high quality. Accordingly, a manufacturer of a vaccine for animals is always required for improvement in a process for production and a process for recovery and purification that enables treatment in large scale and reduction of cost for production.

Patent reference 1: Japanese patent publication No. 2000-279179

Patent reference 2: Japanese patent publication No. 2002-34568

Non-patent reference 1: Garan, J. E. et al., (1990) Infect. Immun., 58. p.3116-3121

Non-patent reference 2: Makino, S. et al., (1998) Microb. Pathog. 25, p.101-109

Non-patent reference 3: Imada, Y. et al. (1999) Proc. Jpn. Pig. Vet. Soc. 34, p.12-

Non-patent reference 4: Imada, Y. et al. (1999) Infect.

Immun. 67 (9), p.4376-4382

## DISCLOSURE OF THE INVENTION

(Technical Problem to be Solved by the Invention)

As described above, when SpaA gene from  
5 Erysipelothrix rhusiopathiae is expressed in E. coli or  
Brevibacillus choshinensis as a host, the protein is  
expressed as a soluble SpaA protein or in admixture of the  
soluble and insoluble proteins, which renders process for  
its production troublesome and does not allow for  
10 expectation of high yield.

The present invention has been accomplished in  
view of necessity on the technical or industrial background  
as described above. Thus, an object of the present  
invention is to provide a process for preparing SpaA or a  
15 shortened form of SpaA ( $\Delta$ SpaA) in which a portion of SpaA  
is deleted, which comprises introducing amino acid  
substitution in the amino acid sequence of SpaA or  $\Delta$ SpaA  
protein so that intrinsically soluble SpaA or  $\Delta$ SpaA protein  
could be expressed as inclusion bodies within the cells of  
20 E. coli, and recovering and purifying the inclusion bodies.

Another object of the present invention is to  
provide a recombinant SpaA or  $\Delta$ SpaA protein obtained by  
said process in high purity.

(Means for Solving the Problems)

25 The present inventors have continued research

assiduously so as to attain the objects as described above and as a consequence have found that there existed clones that may form insoluble inclusion bodies among E. coli cells in which SpaA or  $\Delta$ SpaA protein is expressed, that amino acid substitution occurred at a specific site in the amino acid sequence of SpaA or  $\Delta$ SpaA protein that formed inclusion bodies, and that artificial introduction of said amino acid substitution may allow for accumulation of soluble SpaA or  $\Delta$ SpaA protein as inclusion bodies within the cells. Furthermore, the present inventors have found that soluble SpaA or  $\Delta$ SpaA protein retained immunogenicity even after formation of inclusion bodies to thereby complete the present invention. By way of example, inclusion bodies may be formed when the 69th amino acid in SpaA or  $\Delta$ SpaA protein from SE-9 strain is substituted with glycine; the 214th amino acid is substituted with glutamine; the 278th amino acid is substituted with glycine; the 531st amino acid is substituted with glycine; the 154th and 203rd amino acids are substituted with glycine and threonine, respectively; the 214th and 253rd amino acids are substituted with glutamine and threonine, respectively; or the 69th, 154th and 203rd amino acids are substituted with glycine, glycine and threonine, respectively.

The present invention generally provides a process for preparing a variant of Erysipelothrix rhusiopathiae



surface protective antigen SpaA protein or of a shortened form of SpaA ( $\Delta$ SpaA) in which a portion of SpaA protein is deleted, said variant having immunogenicity and being expressed in E. coli as inclusion bodies, which comprises

5 mutating a gene coding for said SpaA or  $\Delta$ SpaA protein so that amino acid substitution may be introduced in the amino acid sequence of said SpaA or  $\Delta$ SpaA protein, allowing the resulting mutated gene to be expressed in E. coli, and selecting such variants that formed inclusion bodies among

10 the variants expressed. Thus, the process according to the present invention is characterized by that SpaA or  $\Delta$ SpaA protein, of which intrinsically soluble property has made recovery and purification of said protein difficult, may be expressed in E. coli as insoluble inclusion bodies by

15 preparing a variant of SpaA or  $\Delta$ SpaA protein through amino acid substitution that enables expression of said protein as insoluble inclusion bodies to thereby facilitate recovery and purification of said protein.

In one embodiment, the process of the present

20 invention comprises the following steps (A) to (D):

(A) introducing mutation in a gene coding for soluble Erysipelothrix rhusiopathiae surface protective antigen SpaA or  $\Delta$ SpaA protein so that amino acid substitution may be introduced;

25 (B) transforming E. coli cells with an expression

vector containing the resulting mutated gene;

(C) selecting E. coli cells that formed insoluble inclusion bodies among the above transformed E. coli cells; and

5 (D) culturing the selected E. coli cells for recovery of the inclusion bodies within the cells.

To confirm that a variant of recombinant SpaA or  $\Delta$ SpaA protein obtained by the process of the present invention retains a protective activity (immunogenicity) to  
10 Erysipelothrix rhusiopathiae infection, the variant may be further subject to the following steps (E) to (F):

(E) administering the inclusion bodies or the inclusion bodies treated with a solubilizing agent to an animal sensitive to Erysipelothrix rhusiopathiae infection  
15 and then attacking said animal with a virulent strain of Erysipelothrix rhusiopathiae; and

(F) observing survival or death of the animal sensitive to Erysipelothrix rhusiopathiae to thereby assess the presence of a protective activity (immunogenicity)  
20 against Erysipelothrix rhusiopathiae infection.

The process of the present invention is characterized by that SpaA or  $\Delta$ SpaA protein, which is intrinsically soluble, may be converted into its variant that may be expressed in E. coli as insoluble inclusion  
25 bodies to thereby facilitate recovery and purification of

said protein. In accordance with the process of the present invention, for expression of SpaA or  $\Delta$ SpaA protein as insoluble inclusion bodies, a gene coding for SpaA or  $\Delta$ SpaA protein is mutated to introduce amino acid substitution in the amino acid sequence of said SpaA or  $\Delta$ SpaA protein. Among the thus prepared variants of SpaA or  $\Delta$ SpaA protein with amino acid substitution are included those that may be expressed by forming inclusion bodies, which are then selected. Accordingly, mutation introduced in a gene coding for SpaA or  $\Delta$ SpaA protein or amino acid substitution caused in the amino acid sequence of said SpaA or  $\Delta$ SpaA protein may be any mutation or amino acid substitution so far as it results in a variant of SpaA or  $\Delta$ SpaA protein that may be expressed by forming inclusion bodies.

An example of such amino acid substitution includes one or a combination of more than one selected from the group consisting of (1) to (7) as described below:

(1) the 69th amino acid from the N-terminal encompassing the signal sequence is substituted with glycine;

(2) the 154th amino acid from the N-terminal encompassing the signal sequence is substituted with glycine;

(3) the 203rd amino acid from the N-terminal

encompassing the signal sequence is substituted with threonine;

(4) the 214th amino acid from the N-terminal encompassing the signal sequence is substituted with glutamine;

(5) the 253rd amino acid from the N-terminal encompassing the signal sequence is substituted with threonine;

(6) the 278th amino acid from the N-terminal encompassing the signal sequence is substituted with glycine; and

(7) the 531st amino acid from the N-terminal encompassing the signal sequence is substituted with glycine.

Another example of such amino acid substitution includes: the 154th and 203rd amino acids from the N-terminal encompassing the signal sequence are substituted with glycine and threonine, respectively; the 214th and 253rd amino acids from the N-terminal encompassing the signal sequence are substituted with glutamine and threonine, respectively; and the 69th, 154th and 203rd amino acids from the N-terminal encompassing the signal sequence are substituted with glycine, glycine and threonine, respectively.

The amino acid sequence of SpaA or  $\Delta$ SpaA protein may be the sequence as depicted in SEQ ID NO: 2 or the

sequence as depicted in SEQ ID NO: 2 with deletion at its C-terminal wherein a desired amino acid substitution, in particular, those as described above, may be introduced.

In another embodiment, the present invention provides a variant of Erysipelothrix rhusiopathiae surface protective antigen SpaA or  $\Delta$ SpaA protein which is immunogenic and expressed in E. coli as inclusion bodies. The variant of SpaA or  $\Delta$ SpaA protein of the present invention is preferably prepared by the process as described herein. The term "a variant of Erysipelothrix rhusiopathiae surface protective antigen SpaA or  $\Delta$ SpaA protein" as used herein refers to an insoluble protein mutated from a soluble Erysipelothrix rhusiopathiae surface protective antigen SpaA or  $\Delta$ SpaA protein by specific amino acid substitution. The term "immunogenicity" or "immunogenic" means a capacity of inducing production of a protective antibody or a capacity of protecting from Erysipelothrix rhusiopathiae infection.

Yet in another embodiment, the present invention provides a composition comprising as an active ingredient a variant of Erysipelothrix rhusiopathiae surface protective antigen SpaA or  $\Delta$ SpaA protein of the present invention. The variant of SpaA or  $\Delta$ SpaA protein of the present invention contained in said composition is preferably prepared by the process as described herein.

In yet another embodiment, the present invention provides a gene coding for a variant of Erysipelothrix rhusiopathiae surface protective antigen SpaA or ΔSpaA protein which is immunogenic and expressed in E. coli as inclusion bodies. The gene coding for a variant of SpaA or ΔSpaA protein of the present invention is preferably prepared by the process as described herein. The gene coding for a variant of SpaA or ΔSpaA protein of the present invention includes at least one nucleotide substitution as compared to a gene coding for SpaA or ΔSpaA protein. Said at least one nucleotide substitution however should not be a silent mutation but must induce at least one amino acid substitution (point mutation) in SpaA or ΔSpaA protein.

An example of a gene coding for a variant of SpaA or ΔSpaA protein of the present invention includes, for instance, a nucleotide sequence or a nucleotide sequence with deletion of a portion of the 3'-terminal, which includes one or a combination of more than one nucleotide substitution in SEQ ID NO: 1 selected from the group consisting of (1) to (7) as described below:

(1) the 206th nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G;

(2) the 461st nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G;

(3) the 608th nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is C;

(4) the 642nd nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G;

5 (5) the 758th nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is C;

(6) the 833rd nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G; and

10 (7) the 1591st nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G.

Another example of a gene coding for a variant of SpaA or  $\Delta$ SpaA protein of the present invention includes, for instance, a nucleotide sequence or a nucleotide sequence with deletion of a portion of the 3'-terminal, which includes any of nucleotide substitution in SEQ ID NO: 1 selected from the group consisting of (a) to (h) as described below:

(a) the 206th nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G;

20 (b) the 608th nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is C;

(c) the 642nd nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G;

25 (d) the 833rd nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G; and

(e) the 1591st nucleotide in the nucleotide sequence as depicted in SEQ ID NO: 1 is G;

(f) the 461st and 608th nucleotides in the nucleotide sequence as depicted in SEQ ID NO: 1 are G and C, respectively;

(g) the 642nd and 758th nucleotides in the nucleotide sequence as depicted in SEQ ID NO: 1 are G and C, respectively; and

(h) the 206th, 461st and 608th nucleotides in the nucleotide sequence as depicted in SEQ ID NO: 1 are G, G and C, respectively.

In still another embodiment, the present invention provides a method for using a variant of Erysipelothrix rhusiopathiae surface protective antigen SpaA or ΔSpaA protein of the present invention as a vaccine for porcine erysipelas. The variant of SpaA or ΔSpaA protein of the present invention used in said method is preferably prepared by the process as described herein.

Erysipelothrix rhusiopathiae for use in preparing a variant of Erysipelothrix rhusiopathiae surface protective antigen SpaA or ΔSpaA protein of the present invention includes, for instance, Fujisawa strain, Koganai strain for type 1, and Tama 96 strain, SE-9 strain or Shizuoka 63 strain for type 2 but SpaA gene from any strain of Erysipelothrix rhusiopathiae may be used in the present



invention.

(More Efficacious Effects than Prior Art)

In accordance with the present invention, a method for expressing soluble SpaA or  $\Delta$ SpaA protein in E. coli as insoluble inclusion bodies is provided. The expression in  
5 E. coli as inclusion bodies allows for purification of SpaA or  $\Delta$ SpaA protein easily in high purity simply by centrifugation and washing procedures. The thus obtained inclusion bodies of SpaA or  $\Delta$ SpaA protein, after  
10 solubilization, have sufficient purity and immunogenicity for use as a vaccine only if diluted. Thus, a simple and efficient method for preparing a SpaA or  $\Delta$ SpaA protein vaccine is provided. With the use of SpaA or  $\Delta$ SpaA protein obtained by said method, opportunity of Erysipelothrix  
15 rhusiopathiae infection to human may be reduced as compared to a method for preparing an inactivated vaccine or a component vaccine which employs Erysipelothrix  
rhusiopathiae as a starting material. The present invention also evades problems of restoration of  
20 pathogenicity in Erysipelothrix rhusiopathiae, severe side effects found in swine with low antibody titer or SPF swine, and the like.

#### BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows an expression vector for SpaA or  
25  $\Delta$ SpaA protein. a: Plasmid pET11d/SpaA in which a gene

coding for SpaA protein derived from Erysipelothrix rhusiopathiae SE-9 strain is inserted; b: Plasmid pET11d/ $\Delta$ SpaA in which a gene coding for  $\Delta$ SpaA protein is inserted.

5            Fig. 2A shows results of SDS-PAGE performed on SpaA protein derived from Erysipelothrix rhusiopathiae SE-9 strain and  $\Delta$ SpaA protein. M: Marker; Lane 1: culture of E. coli not expressing a foreign protein; Lane 2: culture of E. coli expressing SpaA protein; Lane 3: culture of E. coli  
10            expressing  $\Delta$ SpaA protein.

            Fig. 2B shows results of SDS-PAGE performed on  $\Delta$ SpaA protein derived from Erysipelothrix rhusiopathiae. M: Marker; Lane 1: culture of E. coli not expressing a foreign protein; Lane 2: culture of E. coli expressing  
15             $\Delta$ SpaA protein derived from Fujisawa strain; Lane 3: culture of E. coli expressing  $\Delta$ SpaA protein derived from Tama 96 strain; Lane 4: culture of E. coli expressing  $\Delta$ SpaA protein derived from Koganai strain; Lane 5: culture of E. coli expressing  $\Delta$ SpaA protein derived from SE-9 strain.

20            Fig. 3 shows results of SDS-PAGE performed on soluble and insoluble (inclusion bodies)  $\Delta$ SpaA proteins derived from Erysipelothrix rhusiopathiae SE-9 strain. M: Marker; Lane 1: supernatant of centrifugation of sonicated culture of E. coli expressing soluble  $\Delta$ SpaA protein; Lane  
25            2: precipitates of centrifugation of sonicated culture of E.

coli expressing soluble  $\Delta$ SpaA protein; Lane 3: supernatant of centrifuge of sonicated culture of E. coli expressing insoluble  $\Delta$ SpaA protein; Lane 4: precipitates of centrifugation of sonicated culture of E. coli expressing insoluble  $\Delta$ SpaA protein.

Fig. 4A shows mutated sites and restriction enzyme cleavage sites found in SpaA gene in the plasmids extracted from E. coli transformant cells (three clones; No. 1, No. 2 and No. 3) expressing insoluble (inclusion bodies)  $\Delta$ SpaA protein from comparison with SEQ ID NO: 7.

Fig. 4B shows mutated sites and restriction enzyme cleavage sites found in SpaA gene in the plasmid extracted from E. coli transformant cells (one clone; No. 4) expressing insoluble (inclusion bodies) SpaA protein from comparison with SEQ ID NO: 7.

Fig. 5 shows results of SDS-PAGE performed on soluble and insoluble (inclusion bodies) SpaA proteins derived from Erysipelothrix rhusiopathiae SE-9 strain. M: Marker; Lane 1: supernatant of centrifugation of sonicated culture of E. coli expressing soluble SpaA protein; Lane 2: precipitates of centrifugation of sonicated culture of E. coli expressing soluble SpaA protein; Lane 3: supernatant of centrifugation of sonicated culture of E. coli expressing insoluble SpaA protein; Lane 4: precipitates of centrifugation of sonicated culture of E. coli expressing

insoluble SpaA protein.

Fig. 6 shows results of SDS-PAGE performed on insoluble (inclusion bodies) SpaA and  $\Delta$ SpaA proteins derived from Erysipelothrix rhusiopathiae SE-9 strain after purification. M: Marker; Lane 1: SpaA protein; Lane 2:  $\Delta$ SpaA protein.

#### BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is characterized by a method for expressing SpaA or  $\Delta$ SpaA protein in E. coli as inclusion bodies by substituting an amino acid residue at a specific site in the amino acid sequence of said protein with a specific amino acid, and a process for preparing SpaA or  $\Delta$ SpaA protein by incorporating said method.

##### (1) Cloning of a gene coding for SpaA or $\Delta$ SpaA protein

For Erysipelothrix rhusiopathiae, there are chiefly two serotypes as exhibiting strong pathogenicity to swine which are classified into types 1 and 2. Type 1 includes Fujisawa strain and Koganai strain, whereas type 2 includes Tama 96 strain, SE-9 strain and Shizuoka 63 strain. However, a SpaA gene from any strain of Erysipelothrix rhusiopathiae may be used in the present invention. These cells may be grown with a commercially available culture medium in accordance with the instructions attached thereto. For instance, a fixed amount of the cells may be suspended in Brain Heart Infusion Broth supplemented with 0.1% Tween

80 and the suspension incubated at 37°C for 16 to 48 hours.

A gene coding for SpaA or  $\Delta$ SpaA protein may be obtained by PCR with DNAs extracted from the cells as described above as a template using primers designed from the sequence (SEQ ID NO: 1) described by Imada, Y. et al. (1999) Infect. Immun. 67 (9), p.4376-4382. SEQ ID NO: 1 depicts a full-length nucleotide sequence of SpaA gene derived from Fujisawa strain whereas SEQ ID NO: 2 depicts an amino acid sequence of a full-length SpaA protein derived from Fujisawa strain encompassing a signal peptide. SEQ ID NO: 7 depicts a portion of a full-length nucleotide sequence of SpaA gene derived from SE-9 strain, which corresponds to the sequence of from the 107th to 1854th nucleotide residues in SEQ ID NO: 1. A template DNA may be prepared with a commercially available DNA extraction kit, e.g. Isoplant (NIPPON GENE CO., LTD.), in accordance with the instructions attached thereto. PCR primers are readily available from DNA synthesis contractor services, e.g. QIAGEN, by request, and are preferably added with a sequence of an appropriate restriction enzyme cleavage site at the 5' end. Specifically, synthetic DNAs may be used wherein NcoI site is added to SEQ ID NO: 2 or BamHI site is added to SEQ ID NO: 4 or SEQ ID NO: 5. Primers as depicted in SEQ ID NO: 3 and SEQ ID NO: 5 may be used for amplification of a DNA fragment coding for SpaA protein whereas primers as depicted

in SEQ ID NO: 3 and SEQ ID NO: 4 may be used for amplification of a DNA fragment coding for  $\Delta$ SpaA protein. The resulting DNA fragment coding for SpaA or  $\Delta$ SpaA protein will have addition of twelve nucleotides coding for Met  
5 derived from the restriction enzyme NcoI and the three amino acids at the C-terminal (Ala-Phe-Ala). A DNA fragment coding for  $\Delta$ SpaA protein has a partial SpaA gene up till the 1260th nucleotide and codes for a shortened form of SpaA protein with deletion of 207 amino acid residues at the C-  
10 terminal. Size and site of  $\Delta$ SpaA protein where a portion of SpaA protein is deleted may be determined arbitrarily as occasion demands by altering a position of primer sequences. PCR reaction may be performed with a commercially available LA-Taq kit (TAKARA SHUZO CO.), Advantage HF-2 PCR Kit (BC  
15 Bioscience), etc. in accordance with the protocols attached thereto. A nucleotide sequence of the DNA fragments obtained by PCR may be determined with a DNA sequencer, e.g. ABI PRISM310 Genetic Analyzer (PE Biosystems), after cloning into a TA cloning kit (Invitrogen).

20 The thus obtained gene coding for SpaA or  $\Delta$ SpaA protein is cloned. Specifically, the PCR products as described above are digested with the restriction enzymes NcoI and BamHI, the cleaved fragments are inserted into a suitable plasmid, e.g. pET11d (Novagen), which has  
25 previously been digested with the same restriction enzymes,

and the resulting plasmid is introduced into E. coli. Among the colonies of E. coli, those clones having DNAs coding for the desired protein are selected. For a host E. coli, HB101, JM109, LE392, TB1, BL21 and the like may be used, preferably JM109. A method for introduction of a gene includes electroporation, protoplast, PEG, etc. and any of these techniques may be used. Cloning of a desired gene may be confirmed by purification of the plasmid and determination of the nucleotide sequence. A series of these procedures for genetic recombination may be performed in accordance with a general technique for genetic recombination as described by Sambrook et al., Molecular Cloning, A Laboratory Manual Second Edition, Cold Spring Harbor Laboratory Press, N.Y., 1989. In practice, it may be performed with a commercially available kit in accordance with the instructions attached thereto.

(2) Expression and purification of insoluble SpaA or  $\Delta$ SpaA protein

By making a point mutation at a specific site in the cloned gene coding for SpaA or  $\Delta$ SpaA protein and introducing the resulting gene into E. coli, an intrinsically soluble SpaA or  $\Delta$ SpaA protein can be expressed as insoluble inclusion bodies.

Point mutation may be performed by site-directed mutagenesis. In practice, a commercially available kit may

be used, including Site-Directed Mutagenesis System from Takara (Mutan-Super Express Km, Mutan-Express Km, Mutan-K, etc.), QuickChange Multi Site-Directed Mutagenesis Kit or QuickChange XL Site-Directed Mutagenesis Kit from Stratagene,  
5 or GeneTailor Site-Directed Mutagenesis System from Invitrogen, in accordance with the instructions attached thereto. Point mutation may also be produced by replacing a nucleic acid fragment of a suitable size in which point mutation has been introduced.

10 Alternatively, as nucleotide substitution of unspecified numbers at unspecified sites may occur in amplified genes at some rate when normal PCR is performed, this may be utilized for introduction of nucleotide substitution. If substituted nucleotides affect amino acid  
15 codons, amino acid mutation may occur, thus possibility of occurrence of clones that form inclusion bodies. By selecting these clones, the inclusion bodies may be obtained.

A soluble SpaA or  $\Delta$ SpaA protein is expressed in E. coli as insoluble inclusion bodies by e.g. substitution of  
20 the 69th amino acid from the N-terminal encompassing the signal sequence with glycine; substitution of the 154th amino acid with glycine; substitution of the 203rd amino acid with threonine; substitution of the 214th amino acid with glutamine; substitution of the 253rd amino acid with  
25 threonine; the 278th amino acid with glycine; and/or



substitution of the 531st amino acid with glycine. Thus, point mutation in SpaA gene is performed so that these amino acid substitutions may occur. Inclusion bodies are formed by introducing amino acid mutation at at least one of the sites described above but it is possible that amino acid mutation is introduced at all of these sites insofar as the resulting mutants remain immunogenic. Preferably, point mutation in SpaA gene is performed so that the 69th amino acid of SpaA or  $\Delta$ SpaA protein is substituted with glycine; the 214th amino acid is substituted with glutamine; the 278th amino acid is substituted with glycine; the 531st amino acid is substituted with glycine; the 154th and 203rd amino acids are substituted with glycine and threonine, respectively; the 214th and 253rd amino acids are substituted with glutamine and threonine, respectively; or the 69th, 154th and 203rd amino acids are substituted with glycine, glycine and threonine, respectively.

A region and size of  $\Delta$ SpaA protein, obtained by deletion of a portion of SpaA protein, is not subject to restriction insofar as  $\Delta$ SpaA protein remains immunogenic and, when amino acid substitution is introduced, is capable of forming inclusion bodies.  $\Delta$ SpaA protein wherein at least about 1/3 of the C-terminal of SpaA protein is deleted may be used in the present invention. Preferably,  $\Delta$ SpaA protein comprises 420 amino acid residues from the N-terminal

encompassing the signal sequence with deletion of 207 amino acids at the C-terminal.

Alternatively, it is also possible to conversely transform insoluble SpaA or  $\Delta$ SpaA protein into soluble SpaA or  $\Delta$ SpaA protein by introducing amino acid substitution in a converse manner to those described above. Thus, in accordance with the process of the present invention, either protein of soluble or insoluble SpaA or  $\Delta$ SpaA may unrestrictedly be obtained as occasion demands.

Expression of the gene coding for SpaA or  $\Delta$ SpaA protein in which point mutation is performed may be done as described above for cloning of the gene. An expression vector may be commercially available ones and appropriate E. coli is selected as a host. For instance, BL21(DE3) or DH5 $\alpha$ (DE3) for a vector with a T7 promoter; HB101, DH5 $\alpha$  or JM109 for a vector with a tryptophan promoter may be used. Preferably, a combination of pET11d (Novagen) vector, which allows for concomitant cloning and expression of a desired protein, with E. coli BL21 strain may be used.

Recombinant E. coli expressing SpaA or  $\Delta$ SpaA protein may be screened as described below. In the presence of an expression inducer (in case of expression system as used in the present invention, IPTG is utilized), the cells cultured and grown are collected by centrifugation at low speed and suspended in an amount of distilled water. The

cells are disrupted by sonication or with a homogenizer such as French Press, Manton Gallinger and are centrifuged at high speed (15,000 rpm, 15 minutes) to recover inclusion bodies in precipitates. Distilled water may appropriately be added with a surfactant (e.g. Triton X100), a chelating agent (e.g. EDTA), lysozyme, etc. Again, the precipitate is suspended in a suitable amount of distilled water and an amount of the suspension is applied to SDS-polyacrylamide gel electrophoresis. After staining with Coomassie Brilliant Blue, expression of SpaA or  $\Delta$ SpaA protein is confirmed by molecular size and stained image. An amount of the formed inclusion bodies may be determined by comparing amounts of SpaA or  $\Delta$ SpaA protein in supernatant and in precipitates after centrifugation as described above. In accordance with the present invention, about 90% or more of SpaA or  $\Delta$ SpaA protein may be found in precipitates. For confirmation (or detection) of SpaA or  $\Delta$ SpaA protein, procedures based on an antigen-antibody reaction such as ELISA, Western blot, dot blot, and the like may also be used in addition to one based on molecular size. These have been commonly used for detection of a foreign protein expressed in E. coli and any of these may suitably be selected as occasion demands.

For purification of SpaA or  $\Delta$ SpaA protein from the thus obtained E. coli cells expressing SpaA or  $\Delta$ SpaA protein, the method as described in Japanese patent

publication No. 2002-34568 or purification procedures commonly used in protein chemistry such as e.g. centrifugation, salting-out, ultrafiltration, isoelectric precipitation, electrophoresis, ion exchange chromatography, affinity chromatography, hydrophobic chromatography, hydroxyapatite chromatography, or a combination thereof may be used. In accordance with the process of the present invention, 90% or more purity of SpaA or  $\Delta$ SpaA protein may be achieved by treating a culture of E. coli cells expressing SpaA or  $\Delta$ SpaA protein with either or both of an enzyme (e.g. lysozyme) and/or sonication (e.g. sound beam type cell homogenizer), followed by repetition of centrifugation (e.g. 15,000 rpm, 15 minutes) and suspension in a washing buffer (e.g. 20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton X-100).

### (3) Immunogenicity of SpaA or $\Delta$ SpaA protein

Immunogenicity of the thus obtained SpaA or  $\Delta$ SpaA protein may be determined by immunizing mice or other animals, infected with Erysipelothrix rhusiopathiae, with these proteins and challenging the animals with a virulent strain of Erysipelothrix rhusiopathiae. A mode of immunization, e.g. administration route such as subcutaneous, intramuscular or intraperitoneal, term of immunization, etc., may also be determined as commonly used for investigating immunogenicity of a vaccine. More specifically, the

antigenic protein is serially diluted by 5-fold in saline supplemented with 25% (vol/vol) aluminum hydroxide gel to prepare serial dilution which is used for immunization of 5 to 10 mice (ddy, 5 weeks old, female) per dilution by subcutaneous administration. Three weeks after immunization, mice receive intradermal injection of live cells of Fujisawa strain, a virulent strain of Erysipelothrix rhusiopathiae, and survival or death of mice is observed for 10 days. Immunizing effects of the antigenic protein may be assessed by a median protective dose (PD50).

SpaA or  $\Delta$ SpaA protein of the present invention, after purification in an insoluble form, may be solubilized with a solubilizing agent such as urea, guanidine hydrochloride or arginine hydrochloride, subjected to sterile filtration with a membrane filter etc., and used as materials for preparing a vaccine for protection of sensitive animals such as e.g. wild boar, whales, chickens, turkeys and human from infection with Erysipelothrix rhusiopathiae or other pathogens. The thus prepared SpaA or  $\Delta$ SpaA protein may be formulated into a pharmaceutical composition by appropriately admixing it with an immunological adjuvant such as aluminum hydroxide, aluminum phosphate, mineral oil or non-mineral oil, a stabilizing agent such as Polysorbate 80, an amino acid or sugars such as lactose or sucrose, and a preserving agent such as

formalin, thimerosal, 2-phenoxyethanol, benzyl alcohol, benzethonium chloride or benzalkonium chloride. When sugars such as lactose or sucrose effective as fillers are added, it may also be formulated as a lyophilized dosage form.

5           The present invention is explained in more detail by means of the following Examples but should not be construed to be limited thereto. In the following Examples, reagents manufactured by Wako Pure Chemical Industries, Ltd., TAKARA SHUZO CO., LTD. or Difco were used unless  
10 otherwise mentioned.

Example 1

(1) Cloning of genes coding for SpaA and ΔSpaA proteins

Erysipelothrix rhusiopathiae, type 1 Fujisawa strain and Koganai strain, and type 2 Tama 96 strain and  
15 SE-9 strain, were cultured in Brain Heart Infusion medium (Difco) supplemented with 0.1% Tween 80 at 37°C for 16 to 48 hours. The culture (about 1.5 to 3.0 mL) was centrifuged. A total genome DNA was extracted from the obtained precipitate (about 0.03 g or more) with a DNA extraction  
20 kit (Isoplat, NIPPON GENE CO., LTD.).

With the total genome DNA as a template, PCR was performed using synthetic primers (a pair of SEQ ID NOs: 3 and 4, a pair of SEQ ID NOs: 3 and 5), prepared on the basis of the nucleotide sequence of SEQ ID NO: 1, and LAPCR Kit  
25 (TAKARA). The reaction solution was kept at 94°C for 3

minutes and then a cycle of 94°C for 60 seconds, 56°C for 30 seconds and 72°C for 60 seconds was repeated for 30 cycles. The primer of SEQ ID NO: 3 was designed for amplifying the region downstream from the 79th nucleotide of SpaA gene wherein NcoI site was added at its 5' end. The primers of SEQ ID NOs: 4 and 5 were designed for amplifying the region up to the 1260th and 1881st (termination codon of SpaA gene) nucleotides of SpaA gene, respectively, wherein BamHI site was added at its 5' end. The PCR provides SpaA gene having the nucleotide sequence of from the 79th to 1881st and the ΔSpaA gene having the nucleotide sequence of from the 79th to 1260th.

The DNA fragments amplified by PCR were dually digested with NcoI and BamHI and the resulting digested products were ligated with a plasmid pET11d (Novagen), which has previously been digested dually with NcoI and BamHI, using T4 DNA ligase. This reaction solution was mixed with E. coli JM109. The mixture was left to stand in ice for several ten seconds, applied to LB agar (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, 1.5% agar, pH 7.0) supplemented with ampicillin 50 µg/ml and left to stand at 37°C overnight. A single colony was inoculated to 1 to 5 mL LB medium supplemented with ampicillin 50 µg/ml and the medium was shook at 30 to 37°C, followed by a routine work-up to extract plasmids containing the gene coding for SpaA and

$\Delta$ SpaA proteins from the cells (Figs. 1-a and 1-b).

## (2) Expression of SpaA and $\Delta$ SpaA proteins

As described in Example 1-(1), the plasmids from each of the different strains were introduced into E. coli BL21(DE3) to give single colonies of transformant. The single colonies were inoculated to 1 to 5 mL LB medium supplemented with ampicillin 50  $\mu$ g/ml and cultured while shaking at 30 to 37°C until OD<sub>600nm</sub> of the culture reached 0.6 to 1.0. A 1/100 volume of IPTG (100 mM) was added to the culture and shake-culture further continued at 37°C for 2 to 3 hours. The culture was mixed with an equivalent volume of 2 $\times$ SDS sample buffer and, after heating at 100°C for 2 minutes, the mixture was applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (nacalai tesque). For all the strains, bands at around 70 and 45 kD were detected, from which stained image expression of SpaA and  $\Delta$ SpaA proteins was confirmed. Fig. 2A shows results of SDS-PAGE for SpaA and  $\Delta$ SpaA proteins derived from SE-9 strain; Fig. 2B shows results of SDS-PAGE for SpaA and  $\Delta$ SpaA proteins derived from Fujisawa strain, Tama 96 strain, Koganai strain and SE-9 strain.

## (3) Form of SpaA and $\Delta$ SpaA proteins

Whether inclusion bodies of SpaA and  $\Delta$ SpaA proteins were formed was investigated as described below.



The culture of Example 1-(2) was centrifuged at 10,000 rpm for 5 minutes and the resulting precipitate was added with a 1/5 to 1/10 volume, based on the culture, of a washing buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton X-100) or distilled water and the cells were suspended to uniformity. To the suspension was added a 1/100 volume of a lysozyme solution (10 mg/ml) for reaction at 30°C for 15 minutes. The mixture under ice-cooling was sonicated with a handy sonicater (manufacturer: Tomy; Model: UR-20P; Output: 5; Time: 15 seconds, 2 to 4 times) and centrifuged at 15,000 rpm for 15 minutes. After supernatant was collected, the precipitate was added with an equivalent volume, based on the sonicated mixture before centrifugation, of a washing buffer and the cells were again suspended to uniformity. To each of the collected supernatant and the precipitate was added an equivalent volume of 2×SDS sample buffer. After heating, each of the mixture was applied to SDS-PAGE and stained with Coomassie Brilliant Blue. If  $\Delta$ SpaA protein was found in the suspension of precipitate, said  $\Delta$ SpaA protein was assessed to form inclusion bodies (Fig. 3). As a result, formation of inclusion bodies was detected in several clones of SE-9 strain (Table 1). Table 1 shows the number of clones that formed inclusion bodies out of the number of clones investigated. ND means "not done".

Table 1

	Clones forming inclusion bodies/Clones expressing $\Delta$ SpaA	Clones forming inclusion bodies/Clones expressing SpaA
Fujisawa strain (type 1)	0/3	ND
SE-9 strain (type 2)	3/30	1/15
Tama 96 strain (type 2)	0/3	ND
Koganai strain (type 1)	0/3	ND

(4) Nucleotide sequence determination in clones forming inclusion bodies

Next, plasmids were extracted from the four clones of SE-9 strain in Table 1 which formed inclusion bodies (No. 1, No. 2, No. 3 and No. 4) and nucleotide sequence of the gene coding for  $\Delta$ SpaA protein was analyzed by entrusting TAKARA BIO INC., custom service center. On comparison with the sequence of SEQ ID NO: 7, the amino acid substitutions due to nucleotide mutations as depicted in Table 2 were observed.

Table 2

Nucl. position	Nucleotide substitution (corresponding amino acid substitution)	Clone
206th	A to G (the 69th glutamic acid to glycine)	No. 2
461st	A to G (the 154th glutamic acid to glycine)	No. 2
608th	T to C (the 203rd isoleucine to threonine)	No. 2
642nd	T to G (the 214th histidine to glutamine)	No. 1
758th	T to C (the 253rd methionine to threonine)	No. 1
833rd	A to G (the 278th aspartic acid to glycine)	No. 3
1591st	A to G (the 531st arginine to glycine)	No. 4

Example 2(1) Protein expression as inclusion bodies by amino acid substitution of  $\Delta$ SpaA protein

Plasmids were constructed wherein DNA fragments  
5 with the nucleotide substitutions as depicted in Table 2  
produced by cleaving the plasmids from the clones forming  
inclusion bodies in Example 1-(4) with suitable restriction  
enzymes were replaced for the corresponding region in the  
gene coding for  $\Delta$ SpaA protein in the plasmids extracted  
10 from the clones (SE-9 strain) expressing soluble  $\Delta$ SpaA  
protein.

Specifically,

(a) the plasmid from the clone No. 1, after dual  
digestion with the restriction enzymes EcoRI and ClaI, was  
15 applied to agarose electrophoresis to isolate and separate  
an EcoRI-ClaI fragment which comprised the gene coding for  
 $\Delta$ SpaA protein ranging from the 587th to 1152nd nucleotides  
(Fig. 4A-1). The obtained fragment was inserted into the  
plasmid from the clones (SE-9 strain) expressing soluble  
20  $\Delta$ SpaA protein previously treated with EcoRI and ClaI to  
thereby prepare a plasmid comprising the gene coding for  
 $\Delta$ SpaA protein in which the 642nd and 758th nucleotides were  
substituted.

In the same manner,

25 (b) a plasmid wherein an EcoRI-ClaI fragment which

comprised the gene coding for  $\Delta$ SpaA protein ranging from the 587th to 1152nd nucleotides (Fig. 4A-b) from the clone No. 3 was inserted (substitution at the 833rd nucleotide);

(c) a plasmid wherein a KpnI-ClaI fragment which  
5 comprised the gene coding for  $\Delta$ SpaA protein ranging from the 266th to 1152nd nucleotides (Fig. 4A-c) from the clone No. 2 was inserted (substitutions at the 461st and 608th nucleotides); and

(d) a plasmid wherein an EcoRI-ClaI fragment which  
10 comprised the gene coding for  $\Delta$ SpaA protein ranging from the 587th to 1152nd nucleotides (Fig. 4A-d) from the clone No. 2 was inserted (substitution at the 608th nucleotide) were constructed.

(e) A plasmid comprising the gene coding for  
15  $\Delta$ SpaA protein in which the 206th nucleotide was substituted was constructed by inserting a KpnI-ClaI fragment which comprises the gene coding for soluble  $\Delta$ SpaA protein ranging from the 266th to 1152nd nucleotides (Fig. 4A-e) into the plasmid from the clone No. 2 treated with KpnI and ClaI.

(f) A plasmid comprising the gene coding for  
20  $\Delta$ SpaA protein in which the 642nd nucleotide was substituted was constructed by site-directed mutagenesis (Takara, Mutan-Super Express Km). Specifically, the plasmid (Fig. 1-b) from the clones (SE-9 strain) expressing soluble  $\Delta$ SpaA  
25 protein was dually digested with EcoRI and HindIII and the

resulting EcoRI-HindIII fragment (967bp), which comprised the gene coding for ΔSpaA protein ranging from the 587th to 1260th nucleotides and a portion of the plasmid pET11d, was cloned into a vector plasmid pKF18k (Takara). Using this  
5 plasmid as a template, PCR was performed as described in Example 1-(1) with the synthetic oligonucleotide for mutagenesis of SEQ ID NO: 6, comprising a sequence of from the 632nd to 657th nucleotides of the gene coding for ΔSpaA protein in which the 642nd nucleotide T was substituted  
10 with G, 5 pmol of selection primers attached to Mutan-Super Express Km Kit from Takara, 5 μl of 10×LAPCR buffer (+Mg<sup>2+</sup>), 8 μl of a mixture of dNTPs, 0.5 μl of an LA-Taq polymerase solution and sterilized distilled water to make a total volume of 50 μl. The resulting PCR solution, after ethanol  
15 precipitation/washing, was cloned into E. coli MV1184 strain (Takara). The obtained plasmid with the mutagenesis was dually digested with EcoRI and BamHI to separate and isolate an EcoRI-BamHI fragment comprising the gene coding for ΔSpaA protein ranging from the 587th to 1260th nucleotides. This  
20 fragment was inserted into the corresponding region of the plasmid from the clones (SE-9 strain) expressing soluble ΔSpaA protein, a starting material, to give a desired plasmid. Likewise, a plasmid comprising the gene coding for ΔSpaA protein in which the 642nd nucleotide T was  
25 substituted by G was constructed for Fujisawa strain and

Tama 96 strain with the same procedure.

The thus obtained plasmids were used for transformation of E. coli BL21(DE3) and the form of the expressed  $\Delta$ SpaA proteins was surveyed. As a result, it was found that every  $\Delta$ SpaA protein from the transformants with any of the plasmids formed inclusion bodies.

(2) Protein expression as inclusion bodies by amino acid substitution of full-length SpaA protein

Plasmids were constructed wherein DNA fragments with the nucleotide substitutions as depicted in Table 2 produced by cleaving the plasmids from the clones forming inclusion bodies in Example 1-(4) with suitable restriction enzymes were replaced for the corresponding region in the gene coding for SpaA protein in the plasmids extracted from the clones (SE-9 strain) expressing soluble SpaA protein.

Specifically,

(a) the plasmid from the clone No. 4, after dual digestion with the restriction enzymes ClaI and BamHI, was applied to agarose electrophoresis to isolate and separate a ClaI-BamHI fragment (Fig. 4B-a) which comprised a sequence of from the 1152nd to 1881st nucleotides (termination codon of the gene coding for SpaA protein) of the gene coding for SpaA protein. The obtained fragment was inserted into the plasmid from the clones (SE-9 strain) expressing soluble full-length SpaA protein previously treated with ClaI and

BamHI to thereby prepare a plasmid comprising the gene coding for SpaA protein in which the 1591st nucleotide was substituted.

(b) the plasmid obtained in Example 2-(1)-(a), after dual digestion with PstI and ClaI, was applied to agarose electrophoresis to isolate and separate a PstI-ClaI fragment (Fig. 4A-f) which comprised a sequence of from the 611th to 1152nd nucleotides of the gene coding for  $\Delta$ SpaA protein. The obtained fragment was inserted into the plasmid from the clones (SE-9 strain) expressing soluble full-length SpaA protein previously treated with PstI and ClaI to thereby prepare a plasmid comprising the gene coding for SpaA protein in which the 642nd and 758th nucleotides were substituted.

The thus obtained plasmids were used for transformation of E. coli BL21(DE3) and the form of the expressed SpaA proteins was surveyed. As a result, it was found that every SpaA protein from the transformants with any of the plasmids formed inclusion bodies (Fig. 5).

### Example 3

#### (1) Purification of SpaA or $\Delta$ SpaA protein forming inclusion bodies

Each of the E. coli cells expressing  $\Delta$ SpaA protein as inclusion bodies obtained in Example 2-(1) and the E. coli cells expressing full-length SpaA protein as

inclusion bodies obtained in Example 2-(2) were cultured. Each 100 ml of the cultures was centrifuged at 10,000 rpm for 5 minutes and the resulting precipitate was added with a 1/5 to 1/10 volume, based on the culture, of a washing  
5 buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton X-100) and the cells were suspended to uniformity. To the suspension was added a 1/100 volume of a lysozyme solution (10 mg/ml) for reaction at 30°C for 15 minutes. The mixture under ice-cooling was sonicated with a sound beam type cell  
10 homogenizer (manufacturer: Branson Sonic Power Co.,; Model: 350; Output: 4; Duty Cycle; 30%; Time: 5 to 15 minutes) and centrifuged at 15,000 rpm for 15 minutes. After supernatant was collected, the precipitate was added with an equivalent volume, based on the sonicated mixture before centrifugation,  
15 of a washing buffer (or sterilized distilled water) and the cells were again suspended to uniformity. The suspension was centrifuged at 15,000 rpm for 15 minutes. After supernatant was collected, the precipitate was added with a washing buffer (or sterilized distilled water). This  
20 procedure of centrifugation/washing was repeated three to five times. For the final washing procedure, the precipitate after centrifugation was suspended in sterilized distilled water. The suspension was again centrifuged at 15,000 rpm for 15 minutes. After supernatant was collected,  
25 the precipitate was suspended in 10 ml of 8 M urea. While



gently shaking at room temperature for 2 hours and then at 5°C for 18 hours, the protein of inclusion bodies was solubilized to give purified SpaA or ΔSpaA protein. The gel after SDS-PAGE was stained with Coomassie Brilliant Blue.

5 Determination with a densitometer demonstrated that SpaA and ΔSpaA proteins thus obtained had 90% or more purity (Fig. 6).

#### (2) Immunogenicity of SpaA or ΔSpaA protein

Immunogenicity of SpaA or ΔSpaA protein was determined as described below. To 4 ml of a solution of SpaA or ΔSpaA protein purified in Example 3-(1) were added  
10 11 ml of saline and 5 ml of aluminum hydroxide gel adjuvant (ALHYDROGEL "85", Superfos Biosector) and the mixture was stirred at room temperature for 2 hours to give a vaccine solution. This vaccine solution was serially diluted by 5-  
15 fold in saline supplemented with 25% (vol/vol) aluminum hydroxide gel to prepare serial dilution which was used for immunization of 10 mice (ddy, 5 weeks old, female) per dilution by subcutaneous administration of 0.5 ml. Three weeks after immunization, mice were challenged by  
20 intradermal injection of about 1,000 bacterial live cells of Fujisawa strain, a virulent strain of Erysipelothrix rhusiopathiae. Survival or death of mice was observed for 10 days and a median protective dose (PD50) of purified SpaA or ΔSpaA protein was determined. As shown in Table 3,  
25 purified SpaA or ΔSpaA protein exhibited extremely high

immunogenicity, i.e. a median protective dose (PD50) of 0.0621 to 0.1885  $\mu\text{g}$ . A median protective dose (50% effective dose) in mice was calculated by Behrens-Karber method as described in Karber G: Beitrag zur kollektiven  
 5 Behandlung pharmakologischer Reihenversuche. Arch. Exp. Path. Pharm., 162:480, 1931; "Saikingaku Jisshu Teiyo" [Summary Practice in Bacteriology], 5th ed., Ed. by "alumni association of Ikagakukenyusho" [Medical Science Laboratory], Maruzen, p.564-565, and in accordance with the  
 10 following equation:

Median protective dose in mice ( $\mu\text{g}$ ) =  $10^m$ ,  $m = X_4 - [(h_0 + h_1)(X_1 - X_0) \times 1/2 + (h_1 + h_2)(X_2 - X_1) \times 1/2 + (h_2 + h_3)(X_3 - X_2) \times 1/2 + (h_3 + h_4)(X_4 - X_3) \times 1/2]$

wherein each of  $X_0, X_1, \dots, X_4$  represents logarithm of the  
 15 respective doses, and each of  $h_0, h_1, \dots, h_4$  represents corresponding effective rate (number of survival/number of challenged) by actual measurement. Logarithm (X) of the respective doses may be given by the equation:  $X = \text{Log}_{10}$  [protein concentration of a sample ( $\mu\text{g}/\text{ml}$ )  $\times$  dose in mice  
 20 (ml)  $\div$  fold of dilution].

Table 3

Purified protein	$\Delta$ SpaA			SpaA	
Site of subst. in SpaA gene	642nd 758th	206th 461st 608th	833rd	1591st	642nd 758th
Protein conc. (mg/ml)	2.30	1.91	2.33	2.11	2.28
Fold of dilution	No. of survival /No. of challenged	No. of survival /No. of challenged	No. of survival /No. of challenged	No. of survival /No. of challenged	No. of survival /No. of challenged
625	10/10	10/10	10/10	10/10	10/10
3125	9/10	8/10	10/10	10/10	10/10
15625	5/10	0/10	4/10	4/10	6/10
78125	0/10	0/10	0/10	0/10	0/10
Median protective dose in mice ( $\mu$ g)	0.0864	0.1885	0.0875	0.0793	0.0621

## INDUSTRIAL APPLICABILITY

In accordance with the present invention, a process for preparing a soluble SpaA or  $\Delta$ SpaA protein in E. coli as insoluble inclusion bodies is provided. Application of the process of the present invention to a process for preparation of a soluble protein allows for establishment of a process for preparing SpaA or  $\Delta$ SpaA protein at a practical level, which ensures stable provision of SpaA or  $\Delta$ SpaA protein in the commercial market. A recombinant SpaA or  $\Delta$ SpaA protein obtained by the process of the present invention retains immunogenicity equivalent

to that of the original soluble protein and may be used as materials for preparing a vaccine to Erysipelothrix rhusiopathiae infection alone or in admixture with various additives such as a stabilizing agent, a protective agent, a preserving agent, and the like. It may also be used as an antigen for preparing a monoclonal/polyclonal antibody or as research materials for investigating binding between anti-SpaA or anti- $\Delta$ SpaA antibody and Erysipelothrix rhusiopathiae. As such, SpaA or  $\Delta$ SpaA protein obtained by the process of the present invention would greatly contribute to the medical and research field.